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ORIGINAL ARTICLE

Spectrophotometric, chemometric and chromatographic determination of naphazoline hydrochloride and chlorpheniramine maleate in the presence of naphazoline hydrochloride alkaline degradation product

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Abstract Four accurate and sensitive methods were developed and validated for determination of naphazoline hydrochloride (NAP) and chlorpheniramine maleate (CLO) in the presence of naphazoline hydrochloride alkaline degradation product (NAP Deg). The first method is a spectrophotometric one , where NAP was determined by the fourth derivative (D^4) spectrophotometric method by measuring the peak amplitude at 302 nm, while CLO was determined by the second derivative of the ratio spectra (DD^2) spectrophotometric method at 276.4 nm. The second method is a chemometric-assisted spectrophotometric method in which partial least squares (PLS-1) and partial component regression (PCR) were used for the determination of NAP, CLO and NAP Deg using the information contained in their absorption spectra of ternary mixture. The third method is a TLC-densitometric one where NAP, CLO and NAP Deg were separated using HPTLC silica gel F254 plates using ethyl acetate:methanol:ammonia: (8:2:0.5, by volume) as the developing system followed by densitometric measurement at 245 nm. The fourth method is HPLC method where NAP, CLO and NAP Deg were separated using ODS C_{18} column and a mobile phase consisting of 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v) delivered at 1.5 mL min^{-1} followed by UV detection at 265 nm. The proposed methods have been successfully applied to the analysis of NAP and CLO in pharmaceutical formulations without interference from the dosage form additives and the results were statistically compared with a reported method.

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1. Introduction

Naphazoline hydrochloride (NAP) Fig. 1a; [2-(1-naphthylmethyl)-2-imidazoline monohydrochloride] is a sympathomimetic, which belongs to the imidazole group. It is used as a vasoconstrictor of a relatively long-lasting action that acts on the α receptors of the vascular smooth muscle.[1]

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Chlorpheniramine maleate (CLO) Fig. 1b; [3-(p-chlorophenyl)-3-(2-pyridyl)-*N*,*N*-dimethylpropylamine] is a powerful H_1-receptor antagonist (anti-histamine), widely used for symptomatic relief of common cold, allergic rhinitis and conjunctivitis, with weak sedative properties.[2]

Several methods were reported for the determination of NAP either alone or in the presence of other drugs including spectrophotometric,[3–6] fluorimetric,[7,8] liquid chromatographic,[9–11] gas chromatographic,[12] TLC-densitometric[5] methods. CLO was determined by spectrophotometric,[5,13–15] liquid chromatographic,[11,16] TLC-densitometric,[6,17,18] polarographic,[19] fluorimetric,[20] nuclear magnetic resonance spectrometric,[21] complexometric,[22–24] and potentiometric[25] methods either alone or in the presence of other drugs.

The binary mixture of NAP and CLO is used for their decongestant and antihistaminic effects. 1-Naphthalene acetic acid (NAP Deg) Fig. 1c, is the alkaline degradation product of NAP obtained after the reflux of NAP with 1 N sodium hydroxide for 2 h.[26] NAP Deg is also one of the potential impurities of NAP in USP[27] and BP.[28] So the determination of NAP in the presence of NAP Deg was necessary. On the other hand CLO was found to be stable against hydrolysis.[29]

Reviewing literature on hand, few methods were found for the determination of NAP and CLO in their binary mixture.[5,15] No analytical methods have been described for the determination of NAP, CLO in the presence of NAP Deg, therefore, the aim of this work is to develop accurate, sensitive, rapid and precise methods for the selective determination of NAP and CLO in their binary mixture in bulk powder, in laboratory prepared mixtures, in their pharmaceutical formulations and in the presence of NAP deg.

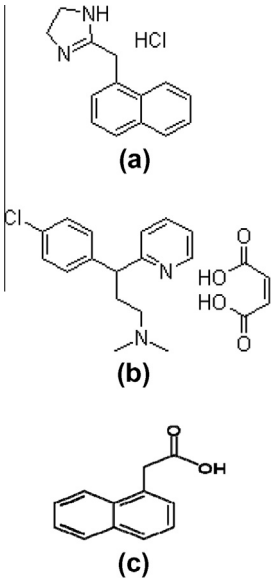

Figure 1 Chemical structure of (a) naphazoline hydrochloride, (b) chlorpheniramine maleate and (c) naphazoline hydrochloride degradation product.

2. Materials and Methods

2.1. Instruments

1. A double beam UV–visible spectrophotometer (SHIMADZU, Japan) model UV-1601 PC with quartz cell of 1 cm path length, connected to IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7. The spectral bandwidth was 2 nm and wavelength-scanning speed, 2800 nm/min.
2. UV lamp with a short wavelength of 254 nm (USA).
3. PLS-Toolbox for use with MATLAB® 6.5.
4. TLC scanner 3 densitometer (Camag, Muttenz, Switzerland).The following requirements are taken into consideration:
   - Slit dimensions: 5×0.2 mm.
   - Scanning speed: 20 mm/S.
   - Spraying rate: 10 s μL^{-1}.
   - Data resolution: 100 μm/step.
5. TLC plates (20×10 cm) coated with silica gel 60F254 (Merck, Germany).
6. Sample applicator for TLC Linomat IV with 100 μL syringe (Camag, Muttenz, Switzerland).
7. Shimadzu class-LC 10 AD liquid chromatograph supplied a UV–visible spectrophotometer detector, a degasser (DGU-3A) and a data processor (C-R4A) all

Table 1 The concentrations of naphazoline HCl, chlorpheniramine maleate and naphazoline HCl degradation product used in the calibration and validation sets.

Mix No.	NAP ($\mu g\ mL^{-1}$)	CLO ($\mu g\ mL^{-1}$)	NAP Deg ($\mu g\ mL^{-1}$)
1	15.00	15.00	9
2	10.00	10.00	5
3	5.00	15.00	7
4	10.00	5.00	9
5	15.00	5.00	5
6	5.00	25.00	5
7	5.00	10.00	13
8	25.00	25.00	7
9	10.00	15.00	13
10	25.00	10.00	9
11	15.00	10.00	7
12	10.00	20.00	7
13	10.00	25.00	11
14	20.00	20.00	13
15	25.00	15.00	11
16	20.00	25.00	9
17	15.00	25.00	13
18	25.00	5.00	13
19	25.00	20.00	5
20	5.00	5.00	11
21	20.00	15.00	5
22	5.00	20.00	9
23	15.00	20.00	11
24	20.00	10.00	11
25	20.00	5.00	7

The concentrations of mixtures used in the validation set are highlighted.

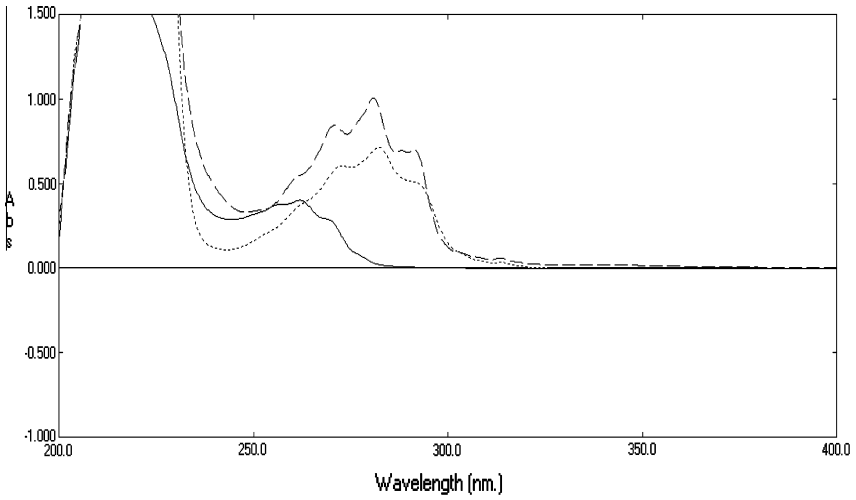

Figure 2 Zero-order absorption spectra of 30 μg mL^{-1} of naphazoline HCl (----), 30 μg mL^{-1} of chlorpheniramine maleate (—) and 20 μg mL^{-1} naphazoline HCl degradation product (. . ..) using methanol as a solvent.

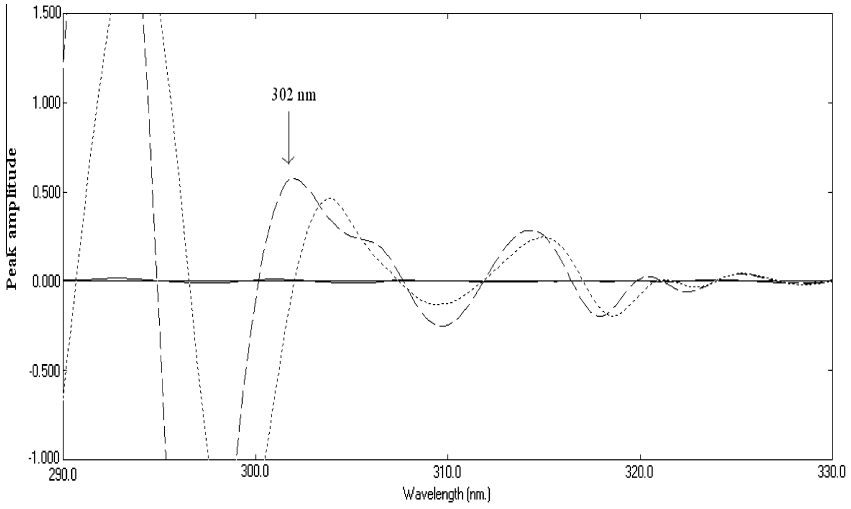

Figure 3 Fourth derivative absorption spectra of 30 μg mL^{-1} of naphazoline (----), 30 μg mL^{-1} of chlorpheniramine maleate (—) and 20 μg mL^{-1} naphazoline HCl degradation product (. . ..) using methanol as a solvent.

from Shimadzu, Kyoto, Japan. The analytical column was a Phenomenex C18-ODS (Shimadzu, Kyoto, Japan), 25 cm × 4.6 mm I.D., 5 μm particle size protected by a C18 guard column.

2.2. Materials

2.2.1. Authentic samples

Naphazoline hydrochloride and chlorpheniramine maleate were kindly supplied by Egyptian international pharmaceutical industries CO. (E.I.P.I.CO) (10th of Ramadan City, Egypt). Their purity was found to be 98.82% and 98.58%, respectively according to the reported spectrophotometric method.[5] 1-Naphthalene acetic acid (NAP Deg) was kindly supplied by Sigma–Aldrich., Germany. Its purity was reported to be 99.39% according to the company's analysis certificate.

2.2.2. Pharmaceutical formulations

Nostamine® eye/nose drops (Batch No. 1101185) labeled to contain 0.5 mg mL^{-1} naphazoline hydrochloride and 0.5 mg mL^{-1} chlorpheniramine maleate is from Egyptian Int. Pharmaceutical Industries Co. (E.I.P.I.CO), 10th of Ramadan City, Egypt.

Neozoline® eye/nose drops (Batch No. 102301) labeled to contain 0.5 mg mL^{-1} naphazoline hydrochloride and 0.5 mg mL^{-1} chlorpheniramine maleate is from Amoun Pharmaceutical Co., El-Obour city, Cairo, Egypt.

Prisoline® eye/nose drops (Batch No. 16237) labeled to contain 0.5 mg mL^{-1} naphazoline hydrochloride and 0.5 mg mL^{-1} chlorpheniramine maleate is from Kahira Pharm. & Chem. Ind. CO., Cairo, Egypt.

2.2.3. Chemicals and solvents

Ethyl acetate, 33% ammonia solution, methanol, KH_2PO_4, phosphoric acid and acetone from (El NASR Pharmaceutical

Table 2 Determination of naphazoline HCl and chlorpheniramine maleate in laboratory proposed mixtures by the proposed spectrophotometric methods.

% NAP Deg from NAP taken Conc.	NAP and CLO ratio	D^4 method At 302 nm NAP			DD2 method At 276.4 nm CLO		
		Taken μg mL^{-1}	Found μg mL^{-1}	Recovery* %	Taken μg mL^{-1}	Found μg mL^{-1}	Recovery* %
10	(1:1)[a]	27	26.44	97.93	27	26.35	97.58
20	1:2	20	19.75	98.77	40	38.96	97.39
30	2:1	20	20.04	100.20	10	9.96	99.60
40	2:3	10	10.56	103.65	15	14.87	99.15
50	3:2	15	16.65	110.97**	10	9.74	97.43
60	3:4	9	10.23	113.72**	12	11.71	97.61
70	4:3	12	13.90	115.85**	9	8.55	95.04**
80	4:5	10	11.62	116.23**	12.5	11.98	95.83**
90	5:4	12.5	14.56	116.46**	10	9.47	94.68**
Mean ± S.D.				100.14 ± 2.522			98.13 ± 0.981

[a] The dosage form ratio.
* Average of three determinations.
** Rejected value.

Chemicals Co., Abu-Zabaal, Cairo, Egypt). Methanol HPLC grade (E. Merck, Germany).

2.3. Standard solutions

2.3.1. Stock standard solutions

Stock standard solutions of NAP, CLO and NAP Deg (1 mg mL^{-1}) were prepared by accurately weighing 100 mg of pure powder of each into three separate 100-mL volumetric flasks, dissolved in and diluted to the volume with methanol (in spectrophotometric, chemometric and HPTLC-densitometric methods) and with methanol:0.1 M KH_2PO_4 (55:45 v/v) (in HPLC method).

2.3.2. Working standard solutions

Working standard solutions of NAP, CLO and NAP Deg (100 μg mL^{-1}) were prepared by transferring 10 mL of stock standard solution of NAP, CLO and NAP Deg (1 mg mL^{-1}) into three separate 100-mL volumetric flasks, diluted to the volume with methanol (in spectrophotometric, chemometric and HPTLC-densitometric methods) and with methanol:0.1 M KH_2PO_4 (55:45 v/v) (in HPLC method).

2.4. Procedures

2.4.1. Derivative and derivative ratio spectrophotometric methods

2.4.1.1. Construction of calibration curve. Into two separate sets of 10-mL volumetric flasks, different aliquots containing 50–400 and 50–450 μg of NAP and CLO, respectively, were accurately transferred from their working solutions; the volume was then completed with methanol. The zero order absorbance of each set was recorded in the range of 200–400 nm. For the determination of NAP, the fourth derivative (D^4) curves were recorded using $\Delta\lambda = 8$ and the scaling factor = 1000, the amplitude values at 302 nm were recorded. For the determination of CLO the second derivative of the ratio spectra (DD^2) was recorded using 30 μg mL^{-1} of NAP Deg as a divisor,

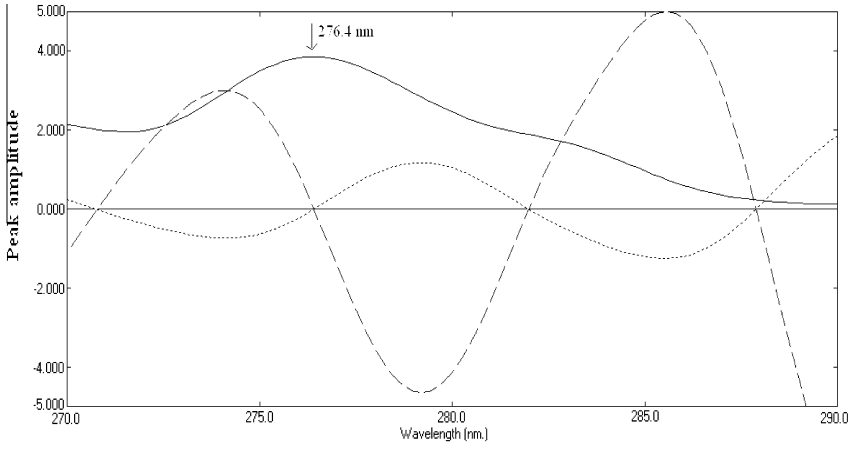

Figure 4 Second derivative ratio absorption spectra of 30 μg mL^{-1} of naphazoline (----), 30 μg mL^{-1} of chlorpheniramine maleate (—) and 20 μg mL^{-1} naphazoline degradation product (….) using 30 μg mL^{-1} of naphazoline HCl degradation product as a divisor and methanol as a solvent.

Table 3 Recovery % of naphazoline HCl, chlorpheniramine maleate and naphazoline HCl degradation product in the validation set using PLS and PCR methods.

NAP					CLO				NAP Deg				
Taken $\mu g\ mL^{-1}$	PLS		PCR		Taken $\mu g\ mL^{-1}$	PLS		PCR		Taken $\mu g\ mL^{-1}$	PLS		PCR	
	Found $\mu g\ mL^{-1}$	Recovery* %	Found $\mu g\ mL^{-1}$	Recovery* %		Found $\mu g.\ mL^{-1}$	Recovery* %	Found $\mu g\ mL^{-1}$	Recovery* %		Found $\mu g\ mL^{-1}$	Recovery* %	Found $\mu g\ mL^{-1}$	Recovery* %
5	4.93	98.59	4.94	98.80	25	25.05	100.20	25.05	100.20	5	5.08	101.60	5.07	101.40
15	14.92	99.53	14.92	99.47	10	9.93	99.31	9.93	99.30	7	7.01	100.14	7.02	100.29
20	20.43	102.16	20.43	102.15	20	19.82	99.10	19.82	99.10	13	12.64	97.23	12.64	97.23
25	25.53	102.12	25.53	102.12	15	15.03	100.22	15.03	100.20	11	10.82	98.36	10.81	98.27
20	20.14	100.70	20.14	100.70	25	24.72	98.88	24.72	98.88	9	9.00	100.00	9.00	100.00
25	24.97	99.88	24.97	99.90	20	19.90	99.50	19.90	99.50	5	4.94	98.80	4.94	98.80
20	19.87	99.35	19.67	98.35	15	15.02	100.13	15.02	100.13	5	5.07	101.40	5.07	101.40
15	15.27	101.80	15.28	101.87	20	19.91	99.55	19.91	99.55	11	11.15	101.36	11.15	101.36
Mean ± SD		100.39 ± 1.541		100.42 ± 1.593			99.61 ± 0.519		99.61 ± 0.517			99.86 ± 1.605		99.84 ± 1.593

* Average of 3 determinations.

$\Delta\lambda$ = 8 and scaling factor = 1000, the amplitude values at 276.4 nm were measured.

The calibration curves were constructed relating the peak amplitudes against the corresponding drug concentrations and the regression equations were computed.

2.4.1.2. Analysis of laboratory prepared mixtures of NAP, CLO and NAP Deg. Mixtures containing different ratios of NAP, CLO and NAP Deg were prepared and analyzed using the proposed method stated above.

2.4.2. Chemometric-assisted spectrophotometric methods

2.4.2.1. Construction of both training and validation sets. A set of 25 synthetic mixtures with different concentrations of each compound were prepared in methanol in the concentration ranges of 5–25 $\mu g\ mL^{-1}$ for NAP, 5–25 $\mu g\ mL^{-1}$ for CLO and 5–13 $\mu g\ mL^{-1}$ for NAP Deg as shown in Table 1. The absorbance of these mixtures was scanned in the range of 200 and 400 nm at 1 nm intervals with respect to a blank of methanol. The composition of the samples was randomly designed according to a five level three factor experimental design.[30,31] The PLS and PCR models were constructed, the training set absorbance and concentration matrices were used together with PLS-Toolbox 2.0 software for the calculations.

2.4.3. HPTLC-densitometric method

2.4.3.1. Construction of calibration curve. Into a series of 10-mL volumetric flasks, aliquots equivalent to 0.5–4.5, 0.5–4, 0.6–1.8 mg, respectively, were accurately transferred from the standard stock solution of NAP, CLO and NAP Deg (1 $mg\ mL^{-1}$ in methanol), then the volume was completed with methanol. Ten microliters of each solution was spotted as bands of 5 mm width on TLC plates (20 × 10 cm) using a Camag Linomat IV applicator. The bands were applied at 5 mm and 15 mm intervals from the bottom and sides. Linear ascending chromatogram developing to a distance of 9 cm was performed in a chromatographic tank previously saturated for 1 h with the developing mobile phase consisting of ethyl acetate:methanol:ammonia solution (8:2:0.5, by volume) at room temperature. The developed plates were air dried and scanned at 245 nm. The peak areas were recorded and the calibration curve was constructed by plotting the integrated peak area versus the corresponding concentrations as $\mu g\ band^{-1}$ of each component and the regression equations were computed.

2.4.4. HPLC method

2.4.4.1. Construction of calibration curve. Aliquots equivalent of NAP, CLO and NAP Deg were accurately separately transferred from their corresponding stock standard solutions (1 $mg\ mL^{-1}$) into a series of 10-mL volumetric flasks, then the volume was completed with 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v) to obtain the final dilution of 5–37, 5–50 and 5–35 $\mu g\ mL^{-1}$ for NAP, CLO and NAP Deg, respectively. Triplicate 20 µL injections were made for each concentration. Chromatograms were recorded under the following instrumental parameters: the flow rate was 1.5 $mL\ min^{-1}$ at ambient temperature and the effluent was monitored at 265 nm. The separation was done on C_{18} column using 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v) as a mobile phase. The peak area of NAP, CLO and NAP Deg were recorded and the peak area ($\times 10^{-4}$) (using 20 $\mu g\ mL^{-1}$ of each

Table 4 Summary of the results obtained by applying the diagnostic tools for model validation of the chemometric methods.

Validation parameters	NAP		CLO		NAP Deg	
	PLS	PCR	PLS	PCR	PLS	PCR
a – predicted vs. known concentration plot						
1 – slope	1.0141	1.0139	0.9921	0.9921	0.9707	0.9706
2 – intercept	−0.1469	−0.1402	0.0700	0.0700	0.2058	0.2054
3 – correlation coefficient	0.9991	0.9991	0.9997	0.9998	0.9991	0.9990
Residual prediction error	0.68	0.68	0.14	0.14	0.20	0.20
b – RMSEP	0.29	0.29	0.13	0.13	0.16	0.16

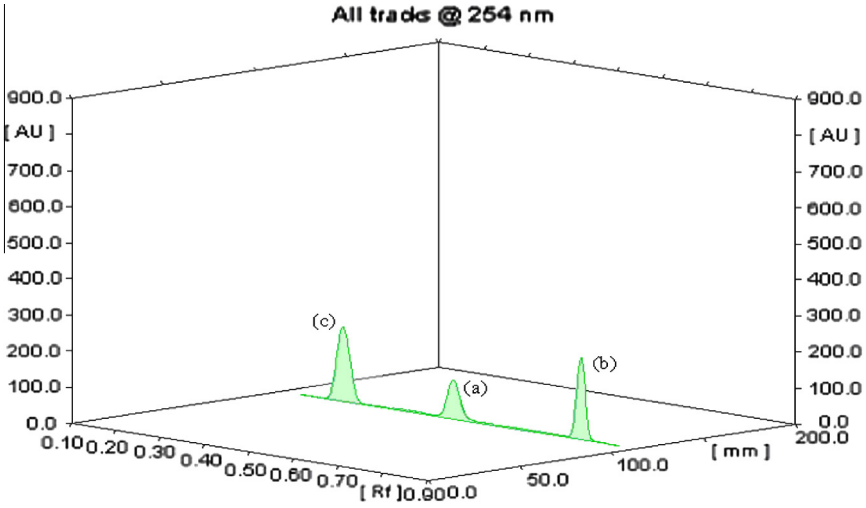

Figure 5 TLC chromatogram of a mixture of, (a) naphazoline hydrochloride, (b) chlorpheniramine maleate and (c) naphazoline hydrochloride degradation product (using: ethyl acetate:methanol:ammonia (8:2:0.5, by volume) as a developing system.

Table 5 Parameters of system suitability of the developed HPTLC-densitometric method.

Parameters	NAP Deg	NAP	CLO
Symmetry factor	1.02	1.01	1.00
Resolution (R_s)	–	2.4	3.1
Capacity factor (K')	0.78	0.55	0.25
Selectivity (α)	–	2.04	1.67

of NAP, CLO and NAP Deg as an external standard) was calculated for each concentration. The calibration curves relating the obtained peak area ratio to corresponding concentration were constructed and the regression equations were then computed.

2.4.5. Application to pharmaceutical formulation

The contents of 10 bottles of each of Nostamine®, Neozoline® and Prisoline® drops were separately mixed well, then 20 mL were transferred accurately into a 100-mL volumetric flask; the volume was completed with methanol to get a 100 μg mL^{-1} working solution of each of NAP and CLO (in spectrophotometric, chemometric and HPTLC-densitometric methods) and with 0.1 M KH_2PO_4: methanol (55:45 v/v) (to obtain 100 μg mL^{-1} working solution for each in the HPLC method).

3. Results and discussion

NAP and CLO are co-formulated in many pharmaceutical formulations, so, it was necessary to determine each of them in the presence of the other. CLO is stable against hydrolysis and no hydrolytic degradation products have been obtained after forced hydrolysis conditions.[29] NAP is liable to hydrolysis after refluxing with 1 N NaOH giving 1-naphthalene acetic acid as a major degradation product after 2 h[26] which is also one of the potential impurities of NAP in both USP[27] and BP[28] so the determination of the binary mixture in bulk powder or in their pharmaceutical formulations without interference of NAP Deg was an analytical task of potential.

3.1. Derivative and derivative ratio spectrophotometric methods

3.1.1. Fourth derivative (D^4) spectrophotometric method

Derivative spectrophotometry offers greater selectivity than does the normal spectrophotometry, as it decreases spectral overlap and allows better resolution.[32]

Fig. 2 shows the severe overlap of the absorption spectra of NAP, CLO, and NAP Deg which prevents their direct simultaneous determination. Different factors affecting separation were studied to optimize resolution of drugs, including solvent effect. Different solvents were tried in order to eliminate interference, e.g., methanol, 0.1 N HCl and 0.1 N NaOH. The best resolution was obtained by applying D^4 for the determination

Table 6 System suitability testing parameters of the HPLC method.

Parameters	Obtained value (NAP)	Obtained value (CLO)	Obtained value (NAP Deg)	Reference value[27]
Resolution	–	2.00	2.75	$R > 0.8$
Relative retention (α)	–	1.31	1.29	>1
Tailing factor(T)	1.02	1.4	1.25	<1.5–2 or <2
Capacity factor(K')	6.07	4.87	3.38	1–10 acceptable
Number of theoretical plates (N)	1744.49	1684.28	1431.00	Increase with efficiency of the separation
HETP	0.014	0.015	0.017	The smaller the value the higher the column efficiency

HETP = height equivalent to theoretical plate, (cm plate^{-1}).

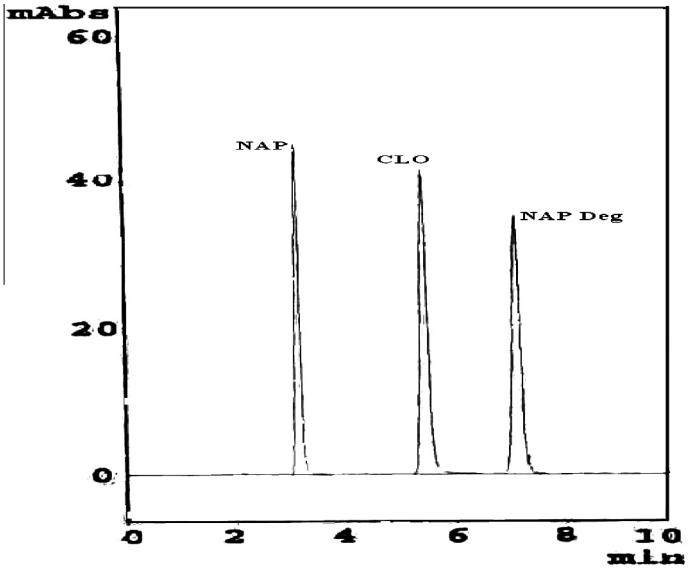

Figure 6 HPLC chromatogram of a mixture of naphazoline hydrochloride, chlorpheniramine maleate and naphazoline hydrochloride degradation product using 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v) as a mobile phase.

of NAP at 302 nm using methanol as solvent. Different parameters affecting D^4 spectra were investigated; different smoothing factor ($\Delta\lambda$) intervals and scaling factor values were tried. The best condition was obtained by using 8 nm as $\Delta\lambda$ interval and the scaling factor, 1000, as shown in Fig. 3.

Linear correlations were obtained between peak amplitudes of NAP at 302 nm in the concentration range of 5–40 μg mL^{-1} from which the regression equation was calculated and found to be:

$$Y = 0.0194C + 0.0089,\ r = 0.9999$$

where Y is the peak amplitude at 302 nm, C is the concentration in μg mL^{-1} and r is the correlation coefficient.

Results described in Table 2 show that this method is selective, valid and applicable for the determination of NAP in the presence of CLO and up to 40% NAP Deg.

3.1.2. Second derivative of ratio (DD^2) spectrophotometric method

Another method for resolving binary mixtures without previous separation is the derivative ratio spectrophotometry, which was developed by Salinas et al.[33]

In this method, the absorption spectra of NAP, CLO and NAP Deg were divided by the spectrum of 30 μg mL^{-1} of NAP Deg (as a divisor), and DD^2 spectra were obtained, where CLO could be determined at 276.4 nm as shown in Fig. 4. Selection of the divisor and its concentration is of great importance, so different concentrations of NAP (10, 15 20 and 30 μg mL^{-1}) and NAP Deg (10, 15, 20 and 30 μg mL^{-1}) were tried as divisors. The best results in terms of signal-to-noise ratio, sensitivity and selectivity were followed using 30 μg mL^{-1} of NAP Deg as a divisor for the determination of CLO.

Linear correlations were obtained between peak amplitudes of CLO at 276.4 nm in the concentration range of 5–45 μg mL^{-1} from which the linear regression equation was computed and found to be:

$$Y = 0.1296C + 0.0131,\ r = 0.9998$$

where Y is the peak amplitude at 276.4 nm, C is the concentration in μg mL^{-1} and r is the correlation coefficient.

Results obtained by applying the suggested DD^2 method in Table 3 show that neither NAP nor NAP Deg influence the determination of CLO concentration in the prepared mixtures containing different ratios of the three components.

Table 7 Determination of naphazoline HCl and chlorpheniramine maleate in Nostamine®, Neozoline® and Prisoline® eye/nose drops by the proposed methods.

Pharmaceutical formulations	Spectrophotometric methods	Chemometric methods		TLC-densitometric method	HPLC method
		PLS	PCR		
Nostamine® eye/nose drops (Batch No. 1101185) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO					
NAP Recovery*% ± S.D.	103.74 ± 1.063	103.92 ± 1.118	104.35 ± 0.852	102.06 ± 1.108	104.60 ± 1.185
CLO Recovery*% ± S.D.	106.89 ± 2.277	104.36 ± 1.284	104.36 ± 1.284	103.82 ± 1.174	105.76 ± 0.434
Neozoline® eye/nose drops (Batch No. 102301) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO					
NAP Recovery*% ± S.D.	98.99 ± 1.230	101.83 ± 0.761	101.59 ± 0.904	98.05 ± 1.114	99.87 ± 1.204
CLO Recovery*% ± S.D.	99.40 ± 0.547	100.52 ± 1.032	101.53 ± 1.039	99.62 ± 0.947	100.46 ± 1.583
Prisoline® eye/nose drops (Batch No. 16237) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO					
NAP Recovery*% ± S.D.	99.72 ± 1.678	–	–	100.23 ± 1.027	98.33 ± 0.657
CLO Recovery*% ± S.D.	101.60 ± 0.742	–	–	98.47 ± 0.961	99.33 ± 0.807

* Average of 6 determinations.

3.2. Chemometric methods

In this method, PLS and PCR chemometric approaches were applied for the determination of NAP, CLO and NAP Deg in their ternary mixture. These chemometric methods were useful in spectral analysis because the simultaneous inclusion of many spectral wavelengths instead of a single wavelength greatly improved the precision and predictive ability.[34] The first step in the simultaneous determination of the three components by chemometric methods involved constructing the calibration matrix for ternary mixture. Seventeen mixtures of NAP, CLO and NAP Deg with different ratios of each component were used for building the calibration model, while eight mixtures were randomly chosen to be used as an external validation set; their concentrations are given in Table 1. Best results were obtained using methanol as a solvent, and rejecting the spectral regions above 300 nm and below 250 nm. In this study, the 'leave one out' cross-validation method was used and the RMSECV values of different developed models were compared. Four factors were found suitable to describe the models.

Results of applying the proposed chemometric methods for the determination of CLO, NAP and NAP deg in the validation set are given in Table 3.

For evaluation of the predictive abilities of the developed model, several diagnostic tools were calculated: predictive versus actual concentration plot (model and sample diagnostic); and root mean square error of prediction (RMSEP) (model diagnostic) as shown in Table 4.

3.3. HPTLC-densitometric method

This technique offers a simple way to quantify directly on the TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing the peak area of the unknown band to a standard curve from reference materials chromatographed simultaneously under the same condition.[35] It is worth mentioning that upon trying the TLC-densitometric method[5] found in the literature, poor resolution of NAP Deg was observed.

The proposed method is based on the difference in the migration rate of the three components, the R_f values were found to be 0.22 for NAP Deg, 0.45 for NAP and 0.75 for CLO (Fig. 5).

The calibration curves were constructed by plotting the peak area ($\times 10^{-4}$) versus the concentration in the ranges of 0.5–4.5, 0.5–4, and 0.6–1.8 μg band^{-1} for NAP, CLO and NAP Deg, respectively, the concentration was calculated from the following regression equation:

$$Y = 0.1892C + 0.0870r = 0.9998 \text{ for NAP}$$

$$Y = 0.1923C + 0.0599r = 0.9999 \text{ for CLO}$$

$$Y = 0.2229C + 0.0600r = 0.9997 \text{ for NAP Deg}$$

where Y is the peak area ($\times 10^{-4}$), C is the concentration in μg band^{-1} and r is the correlation coefficient.

To improve the separation of bands, it was necessary to investigate the effect of different variables. Studying the optimum parameters for maximum separation was carried out as follows:

Table 8 Application of standard addition technique for the determination of naphazoline HCl and chlorpheniramine maleate in Nostamine®, Neozoline® and Prisoline® eye/nose drops by the proposed methods.

Pharmaceutical formulations	Spectrophotometric methods			Chemometric methods						TLC-densitometric method			HPLC method		
				PLS			PCR								
	Added μg mL^{-1}	Recovery *%	Mean ± S.D.	Added μg mL^{-1}	Recovery *%	Mean ± S.D.	Added μg mL^{-1}	Recovery *%	Mean ± S.D.	Added μg band^{-1}	Recovery *%	Mean ± S.D.	Added μg mL^{-1}	Recovery *%	Mean ± S.D.
Nostamine® eye/nose drops (Batch No.1101185) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO															
NAP	5	98.39	101.19 ± 2.449	5	102.86	100.66 ± 2.413	5	102.16	100.43 ± 2.168	0.5	100.00	100.44 ± 1.389	5	101.00	100.02 ± 1.692
	6	102.22		6	101.05		6	101.14		1	102.00		10	101.00	
	7	102.95		7	98.08		7	98.00		1.5	99.33		15	98.07	
CLO	5	103.21	102.21 ± 1.302	5	98.14	99.79 ± 2.441	5	98.09	100.12 ± 2.289	0.5	102.00	100.84 ± 2.449	5	103.11	102.12 ± 1.564
	6	100.74		6	102.60		6	102.60		0.8	102.50		10	100.32	
	7	102.69		7	98.65		7	99.66		1	98.03		15	102.94	
Neozoline® eye/nose drops (Batch No. 102301) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO															
NAP	5	101.55	99.27 ± 1.991	5	101.02	100.05 ± 1.779	5	100.87	101.09 ± 0.381	0.5	102.16	99.95 ± 1.923	5	99.60	100.54 ± 1.227
	6	97.86		6	101.14		6	100.87		1	99.02		10	100.10	
	7	98.41		7	98.00		7	101.53		1.5	98.67		15	101.93	
CLO	5	98.68	100.53 ± 1.671	5	101.41	100.92 ± 1.892	5	101.41	100.79 ± 2.114	0.5	98.00	98.26 ± 0.427	5	102.40	101.39 ± 0.956
	6	101.93		6	102.53		6	102.53		0.8	98.75		10	100.50	
	7	100.98		7	98.84		4	98.44		1	98.02		15	101.27	
Prisoline® eye/nose drops (Batch No. 16237) labeled to contain 0.5 mg/mL NAP and 0.5 mg/mL CLO															
NAP	5	103.36	101.07 ± 2.566	–	–	–	–	–	–	0.5	98.00	98.89 ± 1.541	5	102.00	102.68 ± 0.665
	6	101.57								1	98.00		10	102.70	
	7	98.30								1.5	100.67		15	103.33	
CLO	5	101.07	100.19 ± 1.803	–	–	–	–	–	–	0.5	98.00	100.50 ± 2.291	5	100.80	100.51 ± 1.783
	6	98.12								0.8	102.50		10	98.60	
	7	101.39								1	101.00		15	102.13	

* Average of 3 determinations.

Table 9 Assay parameters and method validation obtained by applying the proposed methods for determination of naphazoline HCl, chlorpheniramine maleate and naphazoline HCl degradation product.

Parameters	Spectrophotometric methods		HPTLC-densitometric method			HPLC method		
	NAP	CLO	NAP	CLO	NAP Deg	NAP	CLO	NAP Deg
Range	5–40 μg mL^{-1}	5–45 μg mL^{-1}	0.5–4.5 μg band^{-1}	0.5–4 μg band^{-1}	0.6–1.8 μg band^{-1}	5–37 μg mL^{-1}	5–50 μg mL^{-1}	5–35 μg mL^{-1}
Linearity								
Slope	0.0194	0.1296	0.1892	0.1923	0.2229	0.1776	0.1347	0.2386
Intercept	0.0089	0.0131	0.0870	0.0599	0.0600	0.0362	0.0021	0.0430
Correlation coefficient	0.9999	0.9998	0.9998	0.9999	0.9997	0.9998	0.9999	0.9999
Accuracy Mean ± S.D.	99.72 ± 1.640	99.88 ± 1.481	100.40 ± 1.143	100.26 ± 0.785	100.45 ± 0.659	100.29 ± 1.258	100.48 ± 1.660	99.97 ± 1.113
Specificity	100.14 ± 2.522	98.13 ± 0.981	–	–	–	–	–	–
Precision (R.S.D. %)								
Repeatability*	1.324	0.897	0.355	0.174	0.753	0.293	0.899	0.415
Intermediate Precision*	1.762	1.554	0.471	0.353	0.658	0.486	0.959	0.818
LOD**	1.46	2.18	0.15	0.14	0.13	0.14	2.11	1.81
LOQ**	4.58	4.84	0.45	0.43	0.49	0.42	4.61	4.09

* The intra-day precision ($n = 3$), average of three different concentrations repeated three times within day. The inter-day precision ($n = 3$), average of three different concentrations repeated three times in three successive days.

** LOD = (SD of the response/slope) × 3.3; LOQ = (SD of the response/slope) × 10.

Different developing systems with different ratios were tried, but a complete separation of NAP, CLO and NAP Deg was achieved by using ethyl acetate:methanol:ammonia solution (8:2:0.5, by volume).

Also different scanning wavelengths were tested like 220 and 265 nm, but on using 245 nm the separated peaks were more sharp and symmetrical with minimum noise.

Parameters including resolution (R_s), peak symmetry, capacity factor (K') and selectivity factor (α) were calculated. The resolution is always above two, the selectivity more than one and accepted values for symmetry factor were obtained, as shown in Table 5.

3.4. HPLC method

A validated isocratic RP-HPLC method with UV detection was developed for the quantitation of NAP, CLO and NAP Deg. It depends on the chromatographic separation of the three components using ODS C_{18} column (25 cm × 4.6 mm i.d. 5 μm particle size) and the mobile phase used consisted of 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v). The mobile phase was delivered at a constant flow rate of 1.5 mL min^{-1}. The injection volume was 20 μL and the effluent was UV-detected at 265 nm.

The relationship between the peak areas (×10^{-4}) and the concentrations of NAP, CLO and NAP Deg were investigated. The linear relationships were tested, resulting in good correlation coefficients in the ranges of 5–37, 5–50 and 5–35 μg mL^{-1} for NAP, CLO and NAP Deg, respectively. The regression equations were computed and found to be:

$$Y = 0.1776C + 0.0362r = 0.9998 \text{ for NAP}$$

$$Y = 0.1347C + 0.0021r = 0.9999 \text{ for CLO}$$

$$Y = 0.2386C + 0.0430r = 0.9999 \text{ for NAP Deg}$$

where, Y is relative peak area, C is the concentration in μg mL^{-1}, and r is the correlation coefficient. The regression equation parameters demonstrated in Table 6 revealed that the suggested method is accurate, precise and specific over the specified ranges.

3.4.1. Optimization of the RP-HPLC method

To optimize the proposed RP-HPLC method, it was necessary to test the effect of different parameters that affect the sensitivity, selectivity and the efficiency of the chromatographic separation.

3.4.1.1. pH effect. The effect of pH on separation was tested by using phosphate buffer at different pH values, it was found that the best pH value was pH = 7. Also the strength of buffer has a great effect on the separation of the three components, 0.1 M KH_2PO_4 was better than 0.025 and 0.05 M.

3.4.1.2. Mobile phase. Different systems were tried to achieve the best chromatographic separation. Complete separation of the three peaks has been obtained upon using 0.1 M KH_2PO_4 (pH = 7):methanol (55:45 v/v) as a mobile phase.

3.4.1.3. Instrumental parameters. Different stationary phases (C_8 and C_{18}) have been tested to optimize the resolution of the three studied components. ODS C_{18} column gave the best

Table 10 Statistical comparison of the results obtained by the proposed methods and the reported method.

Item	NAP					CLO				
	D^4 method	PLS method	PCR method	TLC-densitometric method	HPLC method	Reported method[**5]	DD^2 method	PLS method	PCR method	TLC-densitometric method	HPLC method	Reported Method[**5]
Mean	99.87	100.39	100.42	100.26	100.34	100.11	99.87	99.61	99.61	100.22	101.06	99.88
n	9	8	8	6	7	6	9	8	8	7	6	6
Variance	2.789	2.374	2.303	4.860	2.362	1.257	1.567	2.525	2.303	1.475	3.240	2.556
Student's *t*-test	0.613 (2.145)*	0.462 (2.160)*	0.425 (2.160)*	1.150 (2.201)*	1.084 (2.179)*	–	0.019 (2.145)*	0.396 (2.160)*	0.639 (2.160)*	0.421 (2.179)*	1.201 (2.201)*	–
F-value	2.203 (4.818)*	1.889 (4.876)*	1.832 (4.876)*	3.867 (5.050)*	1.879 (4.950)*	–	1.631 (3.687)*	1.012 (3.972)*	1.109 (3.972)*	1.733 (4.387)*	1.268 (5.050)*	–

* Figures between parentheses represent the corresponding tabulated values of *t* and *F* at $P = 0.05$.
** Reported spectrophotometric method D1, $\Delta\lambda = 4$, at 295.5 nm, for determination of naphazoline HCl and D^2, $\Delta\lambda = 4$, at 261.7 nm for determination of chlorpheniramine maleate using ethanol as a solvent.

separation with the optimum resolution of the three studied components. Different scanning wavelengths have been tested in a trial to enhance the sensitivity of the method. The optimum wavelength for the detection of the three components was at 265 nm. Also different flow rates have been tried to obtain maximum separation within reasonable analysis time, and delivering the mobile phase at 1.5 mL min^{-1} was found suitable for complete separation and good resolution.

The respective compounds were well separated at reasonable retention time. Where, NAP was eluted at 3.93 min, CLO after 5.13 min and NAP Deg after 6.62 min as shown in Fig. 6.

The suggested methods are valid and applicable for the analysis of NAP and CLO in their pharmaceutical formulations (Nostamine®, Neozoline® and Prisoline® drops) (except for the chemometric method which was unable to determine the proposed drugs in Prisoline® drops due to interference from additives) as shown in Table 7. Furthermore, the validity of the proposed methods was assessed by applying the standard addition technique, which showed accurate results and there was no interference from eye drop excipients as shown in Table 8.

Method validation was performed according to USP guidelines[27] for all the proposed methods. Table 9 shows results of accuracy, repeatability and intermediate precision of the derivative and derivative ratio spectrophotometric, HPTLC-densitometric and HPLC methods.

Table 10 shows a statistical comparison of the results obtained by the proposed methods and the reported method.[5] The calculated *t* and *F* values are less than the theoretical ones indicating that there is no significant difference between the proposed methods and the reported method with respect to accuracy and precision.

4. Conclusion

No reported method has been reported for a simultaneous determination of NAP and CLO in the presence of NAP Deg whose UV spectra display complete overlap with NAP and CLO. So the aim of this work was to develop recent, simple, sensitive and validated stability indicating methods for the determination of the two proposed drugs in their binary mixture and in the presence of NAP Deg.

There is only one reported spectrophotometric method concerned with the determination of NAP and CLO in their binary mixture, upon trying this method, it failed to determine either NAP or CLO in the presence of NAP Deg, on the other hand, the proposed spectrophotometric method determined both NAP and CLO successfully in the presence of NAP Deg, so it is considered as a stability indicating method.

5. Conflict of interest

None.

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